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ORIGINAL INVESTIGATION

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Asialo-GM1 and asialo-GM2 are putative adhesion molecules for *Moraxella catarrhalis*

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Abstract *Moraxella catarrhalis* is an important pathogen of respiratory and middle ear infections. We previously reported that the attachment of *M. catarrhalis* to pharyngeal epithelial cells is mediated by ganglioside M2 (GM2). Several sets of adhesins or receptors are involved in such attachment process. In this study, we used the same strains and similar bacterial culture conditions as those in our previous study, and demonstrated by thin layer chromatography that *M. catarrhalis* can also bind toasialo-GM1 (Gg_4Cer) and asialo-GM2 (Gg_3Cer). GalNAcβ1→4Galβ1 is a common sequence in both Gg_4Cer and Gg_3Cer, and in many respiratory bacteria, this sequence acts as a receptor for attachment to host cells. Treatment of human pharyngeal epithelial cells with anti-GM2 and anti-Gg_4Cer antibodies significantlydecreased attachment of *M. catarrhalis* to these cells; however, treatment with anti-Gg_3Cer antibody did not decrease *M. catarrhalis* attachment. Immunofluorescence microscopy revealed that human pharyngeal epithelial cells are positive for GM2 and Gg_4Cer, but not for Gg_3Cer. Our results indicate that Gg_4Cer on human pharyngeal epithelial cells, and Gg_3Cer,possibly on other cells, could serve as molecules for attachment of *M. catarrhalis*.

Keywords *Moraxella catarrhalis* · Attachment · Gangliosides · Anti-ganglioside antibody

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Introduction

The pathogenicity of respiratory infection commences with the colonization of the pharyngeal epithelial cells after successful attachment of bacteria. *Moraxella catarrhalis* is an important organism associated with respiratory and middle ear infections, and attachment has been shown to be a pre requisite for pathogenicity of this bacterium [2]. The emergence of β-lactamase-producing *M. catarrhalis* [15] has made treatment of these infections with conventional β-lactam antibiotics difficult. Resistance in *M. catarrhalis* necessitates a search for new and effective treatment and prevention methods. Antibodies against adhesin and its receptor to block attachment have proved to be a promising approach to prevent infections [4]. Inhibition of glycosphingolipid (GSL) synthesis to deplete the GSLs of the target organism is another novel approach to prevent infections [17]. Therefore, it is essential to elucidate the adhesin and receptor involved in this attachment process.

Attachment of *M. catarrhalis* to human pharyngeal-epithelial cells is mediated by fimbriae [1] and the receptor for this bacterium on pharyngeal epithelial cells resides in the structure of ganglioside GM2 (GM2) [2]. The affinity of a single adhesin molecule for its receptor is considered to be relatively weak because an individual bacterium can have several adhesins that interact with multiple receptor molecules to produce firm binding [5]. Thus, it is possible that other receptors or adhesins are also involved in attachment of *M. catarrhalis*. Accumulating knowledge of the receptor specificity of bacterial adhesins can provide both an explanation of pathogenesis and the potential for developing inhibitors of bacterial attachment to prevent infections. The use of affinity thin layer chromatography (TLC) to screen carbohydrate-based receptors has revealed several features inherent to the receptor-adhesin interaction, including the recognition of internal receptor sequences, low affinity cooperative interactions, and receptor-binding variants of different tropism [23]. Therefore, in

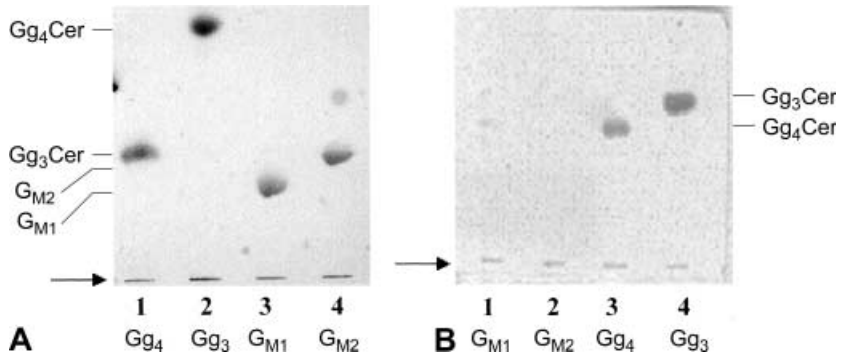

Fig. 1. Binding of*Moraxella catarrhalis* (strain B-88-152) to ganglioside.*Left* Plate (Silica gel 60, Merck, Darmstadt, Germany) sprayed withorcinol stain, after spotting asialo-GM1 (Gg_4Cer), asialo-GM2(Gg_3Cer), ganglioside M1 (GM1) and ganglioside M2 (GM2) in*lanes 1*, *2*, *3* and 4, respectively. *Right* Plate (Polygram,Sil G, Machery-Nagel, Germany) showing reactivity of *M.catarrhalis* with Gg_4Cer (*lane 3*) and Gg_3Cer (*lane 4*), and no reactivity with GM1and GM2 (*lanes 1* and *2*) by immunostaining. There is a faint line in the GM1 lane, which is an artifact.The color of the band is the same as the background color and different fromthat of the immuno reactive bands. In *lane 3* of the orcinol-stained TLC plate, there is only one band for GM1 and no band evidentat the level of Gg_4Cer (lane 1). The *arrow* indicates the site ofthe spotted gangliosides. Both plates were developed inchloroform:methanol:12 mM $MgCl_2$ (5:4:1, v/v/v). Gangliosideswere applied at 500 pmol/lane

the present study we used TLC and a set of glycoconjugates to identify binding molecules for *M. catarrhalis*.

Materials and methods

Bacteria

Strains of *M. catarrhalis*, B-87-34, B-87-69, B-87-75, B-87-94, B-87-133, B-88-83, B-88-152 and Strain F (anon-fimbriated strain), isolated from the sputum of patients with respiratory infections, were used in this study. Strain B-88-152 was mainly used, unless otherwise stated. The bacteria were maintained in Mueller Hinton broth (Becton Dickinson Microbiology Systems, Cockeysville, Md.) containing 5% defibrinated horse blood, and stored at –40°C until use.

Generation of immune serum against *M.catarrhalis*

Whole cell antibody against strain B-88-152 was generated by injecting a rabbit with live organisms, as described previously [1]. A dose of 1 ml bacterial suspension in1 ml Freund's adjuvant (Difco Laboratories, Detroit, MI) was injected each ime in equally divided doses into two subcutaneous and two intramuscularsites. A total of four doses was administered at 2-week intervals. Two weeks after the last injection, blood was collected and the serum was stored at–80°C.

Source of glycolipids

The natural glycolipids used in this study were purified in our laboratory from the following sources: GM1a, GM1b, GD1a, GD1b,GT1b and GalCer, from bovine brain [9, 10, 26]; GlcCer, LacCer, Gb_3Cer andGb_4Cer, from porcine erythrocytes [3]; GM3 from human liver [21]; GM2, from Tay-Sachsbrain [25]; Gg_4Cer andGg_3Cer, from guinea pig erythrocytes [20, 30]; and $IV_3Neu5Ac$-nLc_4Cer, from human red blood cells [13]. nLc_4Cer was prepared from $IV_3Neu5Ac$-nLc_4Cer by sialidase treatment as described previously [19]. Gg_4Cer was prepared by desialylation of GM1 with 1 M formicacid at 80°C for 1 h [30], followed byQ-Sepharose column chromatography to remove acidic glycolipids [9]. The glycoconjugates finally isolated yielded a single spot on high performance silica gel (Polygram, Sil G, Macherey-Nagel, Germany). TLC was developed in two different solvent systems (neutral and basic),chloroform:methanol:12 mM $MgCl_2$ (5:4:1, v/v/v), and chloroform:methanol:2.5 N ammonia (50:40:9, v/v/v), and then stained byorcinol-H_2SO_4 or resorcinol-HCl, as described previously [29]. The structures of gangliosides GM1, GM2 and Gg_3Cer, were identified by nuclear magnetic resonance (NMR) and massspectrometry [10].

The following glycolipids, which were prepared from bovinebrain, were purchased from Sigma Chemical Co. (St. Louis, Mo.): Gg_4Cer, GM1, Gg_3Cer, GM2, GD1a, GD2, GT1b and GQ1b.

TLC for attachment of *M. catarrhalis* with glycolipids

TLC was performed with various glycolipids according to the methods described previously [28] with slight modification. Briefly, glycolipids were separated on a thin-layer plate (Polygram, Sil G, Macherey-Nagel) with a solvent system of chloroform:methanol:water (65:35:8, by volume). After chromatography of variousglycolipids and gangliosides, the plate was dried and then blocked with 1%bovine serum albumin (BSA; Sigma) in phosphate buffer solution (PBS) by shaking at room temperature for 2 h. After five washes with PBS, the plate was incubated overnight at 4°C in *M. catarrhalis* suspension (1×10^8 cfu/ml). After five washes with PBS, the plate was incubated for 2 h at 4°C with antibody against *M. catarrhalis* diluted in 0.1% BSA-PBS. After five washes with PBS, the plate was treated with horseradish peroxidase (HRP)-conjugated protein A(Sigma) diluted with 0.1% BSA-PBS (1:1,000 dilution) for 2 h at 4°C. The plate was then washed five times with PBS and incubated with peroxidase substrate solution. The reaction was observed by examination with the naked eye.

Generation of anti-ganglioside antibodies

Polyclonal anti-GM2 and anti-Gg_4Cer antibodies were prepared as described previously [24, 27]. Briefly, each glycolipid was emulsified with a mixture of 2 ml Freund's complete adjuvant and 3 mg methylated BSA. The emulsion was injected into a rabbit intradermally four times every2 weeks. Six weeks after the last injection, blood was collected and centrifuged to obtain the antiserum. The serum was incubated at 56°C for30 min to inactivate complement. Each antibody was purified on an affinity column conjugated with the corresponding glycolipid antigen after adsorption of each antiserum with GM2 and Gg_4Cer, respectively, as described previously [8]. The purified antibodies were stored at –80°C until use. Rabbit anti-Gg_3Cer antibody was obtained commercially (Matreya Inc., Pleasant Gap, Pa.).

Pharyngeal epithelial cells

Pharyngeal epithelial cells were collected from a healthy adult male subject by scraping the oropharynx with a cotton swab. Cells from

the swab were collected in 1/15 mM PBS, pH 7.2, and washed three times by centrifugation at 80 *g*, each time for 10 min at room temperature. Finally, oropharyngeal cells were adjusted to a density of 2.5×10^4 cells/ml.

Attachment assay

For the adherence assay, cells were treated with different dilutions of anti-GM2, anti-Gg_4Cer and anti-Gg_3Cer serum and normal rabbit serum (NRS) for 30 or150 min at 37°C. *M. catarrhalis* organisms, at a density of 1×10^8 cfu/ml were then mixed with cells andthe adherence assay was performed as described previously [2].

Fluorescence microscopy

Smears were prepared with a Cytospin (Shandon, Astmoor, England) using 0.5 ml of cell suspension with a density of 2.5×10^4 cells/ml. To block nonspecific activity, each smear was incubated with 20 µl of 10% goat serum at room temperature for 30 min. Slides were subsequently incubated with 20 µl anti-GM2, anti-Gg_4Cer or anti-Gg_3Cer antibodies and placed in a moist chamber at 4°C overnight. The slides were rinsed three times with 0.01 M PBS, after which they were treated with 20 µl of secondary antibody (FITC-conjugated goat anti-rabbit IgM, Nordic Immunological Laboratories, Tilburg, The Netherlands) at a dilution of 1:5 and placed in a moist chamber in the dark for 3 h. The slides were againrinsed with 0.01 M PBS to remove unbound antibody. As a negative control, the primary antibodies were substituted with normal rabbit serum or PBS. Slides were coverslipped and examined under a Nikon Microphot-FX microscope (Nikon Company, Tokyo, Japan). The staining intensity was assessed visually and graded semiquantitatively [18] into the following grades: 2+, strong staining intensity; 1+, weak staining intensity; –, absence of staining. The distribution of immunostaining was graded semiquantitatively as diffuse or focal. The grade of immunopositivity was assigned according to the dominant antigenic intensity and distribution observed in each specimen.

Statistical analysis

All data were expressed as mean ± SD.Differences between groups were examined for statistical significance using the Student's *t*–test. A *P* valueless than 0.05 denoted the presence of a statistically significant difference.

Results

Specificity of antiserum against ganglioside

Each anti-GM2 and anti-Gg_4Cer antibody was highly-specific for GM2 and Gg_4Cer, respectively, as assayed by an immunodiffusion test against each glycolipid [24], and TLC immunostaining method [27, 28]. Anti-GM2 antibody reacted with only GM2 in the Ouchterlony (0.1 µmol) and TLC immunostaining method(1 nmol), but not with the structurally related brain gangliosides GM1,GM3, GD1a, GD1b, GT1b, or neutral glycolipids such as Gg_4Cer, glucosylceramide (GlcCer), galactosylceramide (GalCer), lactosylceramide (LacCer), ceramide trihexoside (CTH) and globoside. Anti-Gg_4Cer antibody reacted with Gg_4Cer but not with the above gangliosides or neutral glycolipids, such as GlcCer, GalCer, LacCer, CTH and globoside.

Binding specificity of *M. catarrhalis* with gangliosides on TLC plates

A positive reaction was obtained with Gg_4Cer and Gg_3Cer (. 1), but no reactivity was observed with GalCer, GlcCer, LacCer, Gb_3Cer, Gb_4Cer, nLc_4Cer, GM1a, GM1b, GM2, GM3, GD1a, GD1b and GT1b (Table 1). Using commercially obtained gangliosides, all strains yielded positive reactions with Gg_4Cer and Gg_3Cer only, and no reaction was detected with GM1, GM2, GD1a, GD2, GT1b and GQ1b. Furthermore, no positive reaction was observed with GM2, even at 5 µg/lane.

Effects of antibodies on attachment of *M.catarrhalis*

The number of bacteria attached to pharyngeal epithelial cells was not significantly different after treatment of cells with GM2 or Gg_4Cer antibodies at 1:100 dilution

Table 1. Glycolipids used in thin layer chromatography and their reactivity with *Moraxella catarrhalis*

Glycolipid	Structure	Reactivity
GalCer	Galβ1→Cer	–
GlcCer	Glcβ1→Cer	–
LacCer	Galβ1→4Glcβ1→Cer	–
Gb_3Cer	Galα1→4Galβ1→4Glcβ1→Cer	–
Gb_4Cer (globoside)	GalNAcβ1→3Galα1→4Galβ1→4Glcβ1→Cer	–
nLc_4Cer (paragloboside)	Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→Cer	–
Gg_4Cer (asialo-GM1)	Galβ1→3GalNAcβ1→4Galβ1→4Glcβ1→Cer	+
Gg_3Cer (asialo-GM2)	GalNAcβ1→4Galβ1→4Glcβ1→Cer	+
GM1a	Galβ1→3GalNAcβ1→4(Neu5Acα2→3)Galβ1→4Glcβ1→Cer	–
GM1b	Neu5Acα2→3Galβ1→3GalNAcβ1→4Galβ1→4Glcβ1→Cer	–
GM2	GalNAcβ1→4(Neu5Acα2→3)Galβ1→4Glcβ1→Cer	–
GM3	Neu5Acα2→3Galβ1→4Glcβ1→Cer	–
GD1a	Neu5Acα2→3Galβ1→3GalNAcβ1→4(Neu5Acα2→3) Galβ1→4Glcβ1→Cer	–
GD1b	Galβ1→3GalNAcβ1→4(Neu5Acα2→Neu5Acα2→3) Galβ1→4Glcβ1→Cer	–
GT1b	Neu5Acα2→3Galβ1→GalNAcβ1→ 4(Neu5Acα2→8Neu5Acα2→3)Galβ1→4Glcβ1→Cer	–

Table 2. Attachment of *Moraxella catarrhalis,* B-88-152, to pharyngeal cells treated with NRS, anti-GM2 or anti-asialo-GM1 (anti-Gg_4Cer) antibodies. Data are mean ± SD, numbers in parentheses represent percentages (NRS normal rabbit serum, *anti-GM2*anti-ganglioside M2)

Dilution	Incubation (min)	No. of experiments	NRS	Anti-GM2	Anti-Gg_4Cer
1:100	30	4	26 ± 11.2 (100)	22.8 ± 5.8 (87.7)	29.9 ± 14.7 (115.0)
1:50	30	5	31 ± 9.7 (100)	11.6 ± 3.2[a] (37.4)	26.2 ± 3.8 (84.5)
1:100	150	5	9.7 ± 2.2 (100)	3.7 ± 1.4[b] (38.1)	3.7 ± 0.7 (38.1)

[a] $P < 0.01$ between NRS and anti-GM2, $P < 0.001$ between anti-Gg_4Cer and anti-GM2
[b] $P < 0.005$ between NRS and anti-GM2, $P < 0.005$ between NRS and anti-Gg_4Cer

(Table 2), compared with NRS treatment. However, treatment of cells with GM2 antibody at 1:50 dilution significantly decreased the number of adherent bacteria, compared with cells treated with Gg_4Cer antibody ($P < 0.001$) and NRS ($P < 0.01$). There was no difference in attachment between Gg_4Cer antibody-treated and NRS-treated cells. Extension of the incubation time for pharyngeal epithelial cells and antibodies (1:100 dilution) to 2.5 hr esulted in a significant decrease in attachment, to both Gg_4Cer-and GM2-treated cells ($P < 0.005$) compared with NRS-treated cells (Table 2). However, when cells were treated with anti-Gg_3Cer (1:100 dilution) antibody for 2.5 h, there was no significant decrease in attachment of bacteria to anti-Gg_3Cer-treated cells (9.9 ± 8.3 bacteria/cell), compared with the control (14.7 ± 11.5 bacteria/cell).

Immuno fluorescence microscopy

In the smears of anti-GM2- and anti-Gg_4Cer-treated cells, more than 5–10% and less than 5% of the cells were positively immunostained, respectively. The staining intensity of positive cells in both smears was of 1+ intensity. Immunostaining of GM2 was evident as diffuse and granular staining, while weak and sparse staining for Gg_4Cer was noted in epithelial cells (Fig. 2). When primary antibodies were replaced by NRS and PBS, no specific staining was noted. No difference was observed among smears immunostained with anti-Gg_3Cer antibody, NRS and PBS.

Discussion

The host cell receptors for several bacteria are glycosphingolipid in nature [11, 14, 22, 31]. In the attachment process, several sets of adhesin receptor interactions exist for establishment of strong attachment [7]. In the present study using TLC, we found that *M. catarrhalis* could only bind with Gg_4Cer and Gg_3Cer. GalNAcβ1→ 4Galβ1 is a common sequence for both Gg_4Cer and Gg_3Cer, and for several respiratory bacteria, this sequence may act as an internal sequence for attachment [15]. However, in a previous study using an attachment inhibition assay with commercially available gangliosides, we demonstrated that the receptor for *M. catarrhalis* liesin the sequence of GM2 [2]. In that study,Gg_4Cer could not inhibit attachment even when used at higher concentrations [2]. The receptor-binding specificity of bacteria has been reported to be different depending on the microbes' environment, for example, growth on agar plates or in liquid culture [16]. However, in both of our studies, the same strains of *M. catarrhalis* were used under the same bacterial culture conditions. Moreover, to rule out any differences between commercially obtained and in-house gangliosides, the binding of *M. catarrhalis* to gangliosides from both sources was compared in this study. Interestingly, it was evident with gangliosides from both sources that

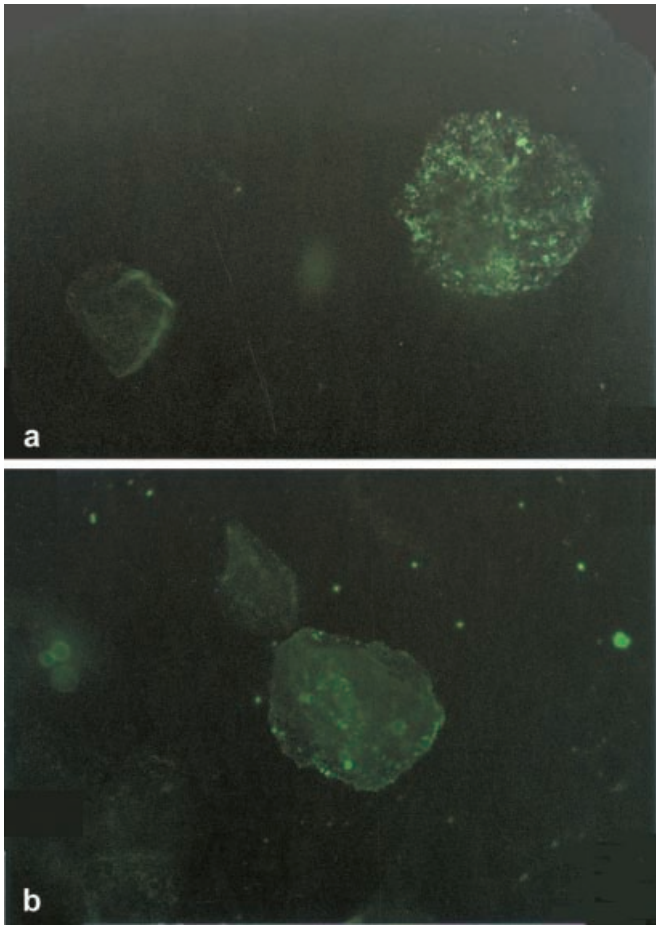

Fig. 2. Immunostaining of ganglioside GM2 (**a**) showing diffuse granular staining on pharyngeal epithelial cells. In contrast to ganglioside GM2 staining, weak and sparse staining for Gg_4Cer (**b**) is noted on pharyngeal epithelial cells

M. catarrhalis can bind with only Gg_4Cer and Gg_3Cer, and even larger amounts of GM2 ganglioside did not show any reactivity. This difference between TLC and attachment inhibition assays was also observed in receptor identification of *Escherichia coli,* where class II adhesin binds equally well to globoside and to Forssman glycolipid or globo A glycolipid when they are presented on thin layer chromatograms. However, on intact cells only globoside is recognized [12]. Conversely, *Burkholderia pseudomallei* exhibited attachment to the Gg_4Cer–Gg_3Cer receptor complex in both TLC and attachment inhibition assay [6].

Logically, the presentation that the bacteria select for optimum colonization should be membrane-dependent presentation of target cells, and the membrane location produces a higher selectivity in binding to glycolipids than when these appear on TLC [12]. In TLC, there is multivalent presentation of the receptor, enabling detection of low-affinity, cooperative multi-site interactions that would escape detection by soluble univalent receptors in attachment inhibition experiments [23]. We propose that multivalent presentation of Gg_4Cer–Gg_3Cer can act as a binding site for *M. catarrhalis*. It is possible that weak binding between *M. catarrhalis* and GM2 occurred on the TLC plate and that the interaction was disturbed by the many washes that are required for the TLC assay [31]. The human pharyngeal cell surface is more complex than the TLC plate; there are many macromolecules present that may enhance or prevent access of bacteria to bind with a particular receptor. Fluorescence microscopy revealed that GM2 and Gg_4Cer were both present on the human pharyngeal epithelial cells and there was a significant decrease of attachment after cells were treated with anti-GM2 and anti-Gg_4Cer antibodies, indicating that these molecules may act as receptors for *M. catarrhalis* on human pharyngeal epithelial cells. However, prolonged incubation of cells with Gg_4Cer antibody was necessary to achieve significant attachment inhibition, indicating that access of antibody to the Gg_4Cer on the cell surface is not easy. The lack of a statistically significant decrease in attachment of *M. catarrhalis* to cells treated with anti-Gg_3Cerantibodies may reflect the unavailability of Gg_3Cer on pharyngeal epithelial cells, as shown by immunofluorescence microscopy. It is possible that Gg_4Cer-Gg_3Cer may be shielded by neighboring molecules in the membrane. Further investigations are required to confirm this observation.

A working model predicts that two or multi-step mechanisms are involved in the attachment process. In the first step, a receptor mediates the target ingand tropism of the bacteria, and in the second step, a receptor establishes a true cell membrane attachment or mediates the penetration into cells. To maintain selectivity, the second step receptors cannot be directly accessible from outside of the cells [12]. Further studies are needed to determine the exact receptors required for the first stage of attachment and the next stage of firm binding of *M. catarrhalis*.

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